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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this Idea Expansion (IDEX) Grant, we propose that targeting IRE1 in endocrine resistant breast cancer cells with N-(4-Phenoxy-phenyl)-2-(5-pyridin-3-yl-2H-[1,2,4]triazol-3-ylsulfanyl)-acetamide (NPPTA; lead compound), or its analogs, will block pro-survival signaling from the UPR and prevent survival (via pro-survival autophagy and an inhibition of apoptosis). We hypothesize that these effects will be mediated in part by the inhibition of XBP1 splicing and its ability to regulate BCL2 family members and NFκB. Furthermore, a combination of NPPTA and AEs will interact synergistically to selectively kill AE resistant breast cancer cells in vitro and in vivo.

15. SUBJECT TERMS

Nothing listed

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INTRODUCTION

In this Idea Expansion (IDEX) Grant, we propose to target IRE1 in endocrine resistant breast cancer cells with N-(4-Phenoxy-phenyl)-2-(5-pyridin-3-yl-2H-[1,2,4]triazol-3-ylsulfanyl)-acetamide (NPPTA; lead compound), or its analogs, will block pro-survival signaling from the UPR and prevent survival (via pro-survival autophagy and an inhibition of apoptosis). We hypothesize that these effects will be mediated in part by the inhibition of XBP1 splicing and its ability to regulate BCL2 family members and NFκB. Furthermore, a combination of NPPTA and AEs will interact synergistically to selectively kill AE resistant breast cancer cells in vitro and in vivo.

KEYWORDS

Breast Cancer, Drug Development, Endocrine Pathogenesis, Endocrine Resistance

ACCOMPLISHMENTS

What were the major goals of the project?

Aim 1: We will use in silico modeling of NPPTA:IRE1 interactions and quantitative structure-activity relationship analyses (QSAR) to develop rationally designed NPPTA analogs with increased potency and optimized pharmacologic properties.

Aim 2: We will determine the ability of NPPTA and its analogs to sensitize responsive breast cancer cells, and re-sensitize resistant cells, to both estrogen withdrawal (analogous to treatment with an AI) and to two different classes of AE (TAM and ICI). These studies will be done initially in vitro, with the strongest candidates being studied in vivo to provide preclinical safety, efficacy, and toxicology data to support later first-in-human studies.

Aim 3: We will explore the mechanism(s) of action of NPPTA and its analogs in inducing cell death, focusing initially on its effects on BCL2 family members and NFκB. We will use high throughput transcriptome analyses to study the effects of NPPTA (or its analogs) on cell survival signaling.

What was accomplished under these goals?

The experiments and findings in the 2nd (2014-2015) year for this study are summarized below:

Using the Reverse Biacore Experiment, in an effort to determine the binding affinity of NPPTA for IRE1alpha we attached the synthesized biotinylated compound to the surface of an avidin-coated surface Plasmon resonance chip. Recombinant protein was flowed over the surface to detect binding. Initial binding was observed but results were not reproducible. Studies are ongoing to develop a reproducible assay.

In an effort to identify the targets for NPPTA, we conducted a kinase screen through DiscoverX using the KINOMEscan™ screening platform. Both NPPTA and JS-1-20 were submitted for the screen.

Kinase assay protocol (DiscoverX): For most assays, kinase-tagged T7 phage strains were grown in parallel in 24-well blocks in an E. coli host derived from the BL21 strain. *E. coli* were grown to log-phase and infected with T7 phage from a frozen stock (multiplicity of infection = 0.4) and incubated with shaking at 32°C until lysis (90-150 minutes). The lysates were centrifuged (6,000 x g) and filtered (0.2μm) to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated small molecule ligands for 30 minutes at room temperature to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer (SeaBlock (Pierce), 1% BSA, 0.05 % Tween 20, 1 mM DTT) to remove

unbound ligand and to reduce non-specific phage binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20 % SeaBlock, 0.17x PBS, 0.05 % Tween 20, 6 mM DTT). Test compounds were prepared as 40x stocks in 100% DMSO and directly diluted into the assay. All reactions were performed in polypropylene 384-well plates in a final volume of 0.04 ml. The assay plates were incubated at room temperature with shaking for 1 hour and the affinity beads were washed with wash buffer (1x PBS, 0.05 % Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05 % Tween 20, 0.5 µM non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR. 134 non-mutant kinases were tested. Two kinases (RIOK2 and STK35) were identified hits for NPPTA. There were no hits for JS-1-20. See results in Table 1.

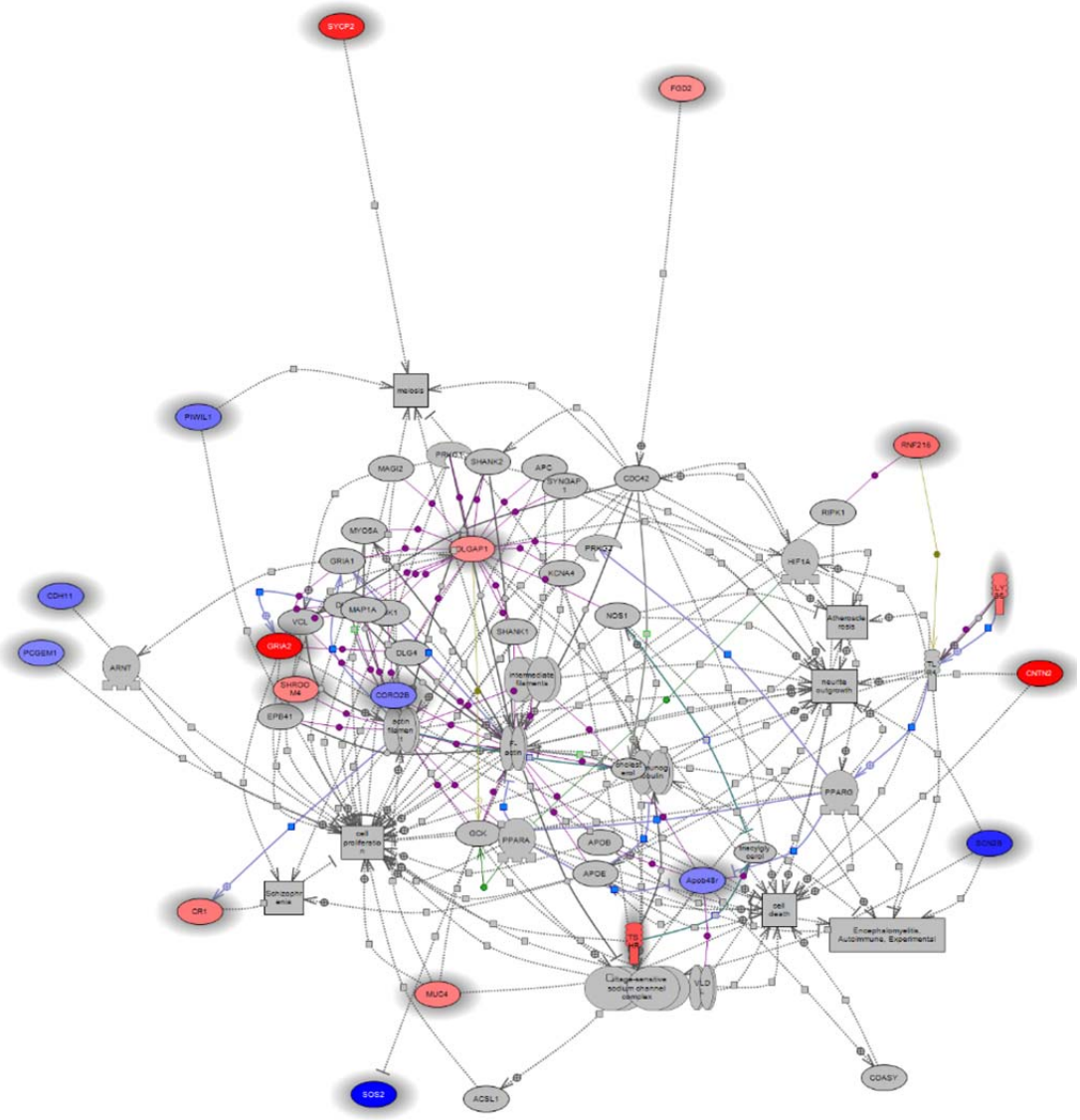

Figure 1: Pathway analysis of differentially expressed genes in breast cancer cell lines treated with 10 mM NPPTA versus vehicle alone.

In addition, to identify the underlying mechanism NPPTA-mediated cell death, we have completed a gene expression array in LCC9 breast cancer cells treated with vehicle alone or 10 mM NPPTA for 72 h (Figure 1). We are in the process of validating the genes that are differentially expressed in NPPTA treated cells. A Reverse Phase Protein Array (RPPA) is underway for the same treatment conditions in both LCC1 and LCC9 cells.

TABLE 1: Kinase screen for NPPTA and JS-1-20

Compound Name	Selectivity Score Type	Number of Hits	Number of Non-Mutant Kinases	Screening Concentration (nM)	Selectivity Score
JS-1-20	S(35)	0	134	10000	0
JS-1-20	S(10)	0	134	10000	0
JS-1-20	S(1)	0	134	10000	0
NPPTA	S(35)	2	134	10000	0.015
NPPTA	S(10)	0	134	10000	0
NPPTA	S(1)	0	134	10000	0

JS20 Salt Synthesis: Synthetic efforts have been made toward creating a more water soluble analog. However due to the amphoteric nature of the 1,2,4-triazole, neither acidic or basic conditions yielded a salt. Synthetic studies are ongoing to resolve this issue.

Prodrug Synthesis: We are also pursuing another method to create a more soluble compound which involves covalently attaching a phosphate group onto NPPTA. This compound should be soluble in water and we are currently working to synthesize this compound.

What opportunities for training and professional development has the project provided?

Dr. Smith is an African-American postdoctoral fellow. Her work on this project was carefully mentored and she was supported throughout with advice and direction scientifically, academic ally and with respect to her career development. Ahreej Eltayeb, an African-American MPH graduate student who also worked on this project, received training in cellular and molecular biology techniques.

How were the results disseminated to communities of interest?

US Appl. No. 14/009,969 as previously reported; nothing additional to report.

What do you plan to do during the next reporting period to accomplish the goals?

As detailed above, efforts towards J20 Salt Synthesis and Prodrug Synthesis are ongoing. We have requested a no-cost extension to provide additional time to complete the *in vivo* testing of NPPTA and its analogs. While one of the analogs of NPPTA, JS-1-20, was tested in a xenograft model with 5 mice, we will expand this study with n=15 mice per group and include an antiestrogen (Faslodex) in combination with JS-1-20. For this, we will need to scale up the synthesis of the compounds and test them *in vitro* before beginning the *in vivo* studies. Furthermore, we will use high throughput transcriptome analysis and protein arrays to study the effects of NPPTA (or its analogs) on tumor survival signaling, particularly the effects on proteins involved in the unfolded protein response (UPR) pathway.

IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

The unfolded protein response (UPR) is a mechanism that is used by some cancer cells to escape therapy induced cell death. In this project, we focused on targeting one essential component of the UPR pathway in endocrine resistant breast cancer: IRE1a. It is known that IRE1a is both an endoribonuclease as well as a kinase. In this funding period, we have found that our compound, NPPTA, may inhibit the kinase activity of IRE1a. We are currently investigating whether the kinase activation of IRE1a is regulated by STK35. Finally, we will further test whether NPPTA or its analog, JS-1-20, can inhibit the growth of human xenografts (tumors).

- **What was the impact on other disciplines?**

  Nothing to Report

- **What was the impact on technology transfer?**

US Appl. No. 14/009,969

- **What was the impact on society beyond science and technology?**

  Nothing to Report

CHANGES/PROBLEMS

Changes in approach and reasons for change

None

Actual or anticipated problems or delays and actions or plans to resolve them

As stated above, we have requested a no-cost extension to provide time to complete the *in vivo* testing of NPPTA and its analogs.

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

PRODUCTS

Publications, conference papers, and presentations

Nothing to Report

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
  - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

    ***Example**:*

Name:	*Robert Clarke, PhD, DSc*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*ORCID:* 0000-0002-9278-0854
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Clarke is the PI of this project and as such is primarily responsible for the overall direction and operation of this application. He directs, coordinates and integrates the proposed studies, prepares reports and manuscripts to disseminate results.*
Funding Support:	*N/A*

Name:	*Ayesha Shajahan-Haq*
Project Role:	*Co-Investigator*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Shajahan-Haq supervises the research assistant and helps perform the experiments specified in the proposal. She also participates in the preparation and presentation of the reports/results and the preparation of manuscripts for publication.*
Funding Support:	*N/A*

Name:	*Jacqueline Smith, PhD*
Project Role:	*Post-doctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*7*
Contribution to Project:	*Dr. Smith is an organic chemist and works closely with Drs. Brown, Clarke, and Shajahan-Haq to design and synthesize NPPTA analogs for the study. She follows up on the in vitro and in vivo studies with NPPTA and its analogs and provides feedback to the bioligists.*
Funding Support:	*N/A*

Name:	*Ahreej Eltayeb*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*The research assistant prepares reagents and buffers, maintains cell cultures, and assists Dr. Shajahan-Haq in the conduct of experiments.*
Funding Support:	*N/A*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to Report

- **What other organizations were involved as partners?**

  Nothing to Report

SPECIAL REPORTING REQUIREMENTS

None

APPENDICES

None.